Synthesis of Oxalic Acid by Enzymes from Lettuce Leaves

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ABSTRACT

A rapid purification of lactate dehydrogenase and glycolate oxidase from lettuce (Lactuca sativa) leaves is described. The kinetics of both enzymes are reported in relation to their possible roles in the production of oxalate. Lettuce lactate dehydrogenase behaves like mammalian dehydrogenase, catalyzing the dismutation of glyoxylate to glycolate and oxalate. A model is proposed in which glycolate oxidase in the peroxisomes and lactate dehydrogenase in the cytosol are involved in the production of oxalate. The effect of pH on the balance between oxalate and glycolate produced from glyoxylate suggests that in leaves lactate dehydrogenase may function as part of an oxalate-based biochemical, pH-stat.

The enzyme glycolate oxidase (EC 1.1.3.1), which catalyzes the oxidation of short chain α-hydroxyacids, was first demonstrated in green leaves by Tolbert et al. (28) and shown to be a flavoprotein by Zelitch and Ochoa (31). Originally, the enzyme was thought not to be capable of oxidizing glyoxylate, but Richardson and Tolbert (22) demonstrated that purified glycolate oxidase from a number of plants catalyzed the oxidation of glyoxylate to oxalate. It is assumed that the substrate is the hydrated form of glyoxylate CH(OH)2COOH, and, as such, it is an α-hydroxyacid analog. The implication that glycolate oxidase is responsible for the in vivo synthesis of oxalate from glyoxylate is paralleled by similar considerations in the case of rat liver (15). On the other hand, mammalian lactate dehydrogenase (EC 1.1.1.27), which can catalyze the dismutation of glyoxylate to glycolate and oxalate (23), has been considered the main enzyme catalyzing the oxidation of glyoxylate to oxalate in leucocytes and erythrocytes (25) as well as in the cytosol of human liver and heart (11).

The demonstration by Betsche et al. (2) that some green leaves contain lactate dehydrogenase raises the possibility that it plays a part in the production of oxalate in leaves. In this paper, we examine this possibility and suggest that the oxidation of glyoxylate to oxalate involves the interaction of lactate dehydrogenase and glycolate oxidase.

MATERIALS AND METHODS

Reagents. Reagents for polyacrylamide gel electrophoresis as well as glyoxylate, oxamate, oxalate, glycolate, and nucleotides were obtained from Sigma. CNBr-activated Sepharose, PBE 94, and poly buffer 96 were obtained from Pharmacia, and [1-13C] glyoxylate from the Radiochemical Centre, Amersham. All other reagents were the highest purity available.

Plant Material. Lettuce (Lactuca sativa) was bought from a local market.

Purification of Glycolate Oxidase and Lactate Dehydrogenase. All operations were carried out at 2°C. Washed and devened lettuce leaves (400 g) were homogenized with 40 g of insoluble Polyclar and 400 ml of K-phosphate (pH 7.4, 0.1 M) containing β-mercaptoethanol (5 mM). Prior to homogenization, the mixture was deoxygenated by bubbling N2. After homogenization, the extract was squeezed through cheese cloth and centrifuged at 23,000g for 25 min. The supernatant was fractionated with (NH4)2SO4, the fraction precipitating between 25% and 55% saturation was collected by centrifuging (23,000g for 20 min), dissolved in 50 ml of Tris-HCl buffer (pH 8.0, 5 mM), and dialyzed against 5 l of the same buffer. The buffer was changed until no (NH4)2SO4 could be detected by Nessler reagent. Any precipitate was removed by centrifuging at 32,000g for 10 min and the clear solution poured on to a DEAE-cellulose column (2.5 × 30 cm) previously equilibrated with the same buffer. When the extract had passed through the column, the column was washed with 50 ml of the buffer used for equilibration. The enzymes were eluted with a linear gradient of KCl (0–0.5 M) in 500 ml of the same buffer, and 5-ml fractions were collected. Glycolate oxidase emerged as a single peak followed by a single peak of lactate dehydrogenase.

Purification of Glycolate Oxidase. The peak fractions were combined and dialyzed for 4 h against K-phosphate (pH 6.3, 50 mM) and any precipitate which formed was removed by centrifuging at 32,000g for 10 min. The clear solution was applied to a column (1.5 × 12 cm) of Sepharose-aminohexyl oxamate previously equilibrated with K-phosphate (pH 6.0, 50 mM). When the extract had passed through the column, the column was washed with a solution of KCl (0.5 M) in the same buffer until pigments present in the extract had reached the bottom of the column. The pigments (and a small amount of glycolate oxidase) were removed by washing the column with 3 bed volumes of buffer without KCl. The enzyme was then eluted with glycine buffer (pH 8.5, 0.1 M).

Purification of Lactate Dehydrogenase. The peak fractions from the DEAE-column were combined and dialyzed for 5 h against K-phosphate (pH 6.8, 50 mM). The solution was clarified by centrifuging (32,000g for 10 min) and NADH (25 mg/100 ml) was added. The solution was then applied to a column (1.5 × 12 cm) of Sepharose aminohexyl oxamate, previously equilibrated with K-phosphate (pH 6.8, 50 mM) containing KCl (0.5 M) and NADH (116 μM). When 10 ml of extract had passed into the column, the column was washed with 30 ml of the equilibrating buffer. This procedure was repeated until all the extract had been applied to the column. After the final wash, the enzyme was eluted by omitting NADH from the buffer.

Affinity Gel. Immobilized Sepharose aminohexyl oxamate was prepared by the method of O’Carra and Barry (18).

Protein Measurement. Protein was measured by the method of Chiappelli et al. (4).

Enzyme Assays.

Glycolate Oxidase. Glycolate oxidase was assayed by a slight modification of the method of Zelitch (30). A stock solution containing Na glycolate (2 mM), 2,6-dichlorophenolindophenol
Oxidase was except 25°C and deoxygenated by min at cuvette (30-55% saturation). Glycolate dehydrogenase (50 mm), N-methylphenazine methosulfate (0.1 mm), 3-(3,5-dimethylthiazol-2-yl)-2,5 di-phenyltetrazolium bromide (1 mm), and 73 mm glycine/NaOH buffer (pH 8.5). For detection of glycolate oxidase, the lactate and NAD* were replaced by glycolate (2 mm).

Chromatofocusing. Chromatofocusing was performed with polybuffer exchanger PBE-94, using Tris-HCl buffer (pH 8.5, 25 mM) as the starter buffer and polybuffer 96 for elution, as described by Pharmacia. This method is particularly effective for the lettuce glycolate oxidase, but because of the high isoelectric point reported for the enzyme from peas (13), the method may require modification when applied to peas and possibly to other plant material.

Determination of Oxalate. The formation of [14C]oxalate from [1-14C]glyoxylate was measured after adding carrier oxalate and precipitating as the calcium salt as described by Hodgkinson and Wilkinson (12). Radioactivity was determined in an Intertechnique scintillation counter.

RESULTS

Purification of Glycolate Oxidase and Lactate Dehydrogenase. The two enzyme activities were separated on DEAE-cellulose (Fig. 1). The best separation was achieved when the fraction precipitating between 30 and 50% saturation of (NH4)2SO4 was used, but a higher recovery of lactate dehydrogenase was achieved using a 25 to 55% (NH4)2SO4 fractionation. A high degree of further purification (36-fold for glycolate oxidase and 1,295-fold for lactate dehydrogenase) was achieved by affinity chromatography on Sepharose aminohexyl oxamate. We have previously used this material to purify lactate dehydrogenase from plants (21), and its use for the purification of glycolate oxidase suggested itself from the report that N-octylxoxamate is a strong inhibitor of mammalian glycolate oxidase (24). The overall purification for both enzymes is given in Table I.

Gel electrophoresis of both purified enzymes indicated that they were homogeneous and no evidence for the presence of isoenzymes was obtained. Occasionally, a small impurity has been noted in the preparation of glycolate oxidase. This impurity was readily removed by repeating the affinity chromatography step or by chromatofocusing, when the enzyme emerged at a pH of 8.0.

Properties of Glycolate Oxidase. Absorption Spectrum. The purified enzyme had an absorption spectrum essentially similar to that reported for the enzyme from spinach (10). The ratio E620/E450 was 7.3 and the characteristic peaks of flavin mononucleotide disappeared when the enzyme was reduced by glycolate under nitrogen (Fig. 2).

Kinetics.

Table 1. Purification of Glycolate Oxidase and Lactate Dehydrogenase from Lettuce Leaves

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Enzyme Activity</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
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<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>units/ml</td>
<td>units/mg</td>
<td>-fold</td>
<td>%</td>
</tr>
<tr>
<td>A. Purification of glycolate oxidase</td>
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<td></td>
<td></td>
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<tr>
<td>Extraction</td>
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<td>0.52</td>
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<td>B. Purification of lactate dehydrogenase</td>
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<td></td>
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</tr>
<tr>
<td>Extraction</td>
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FIG. 3. Effect of pH on the activity of glycolate oxidase (A) and lactate dehydrogenase (B). A: O—O, glycolate to glyoxylate; •—•, glyoxylate to oxalate. B: O—O, glycolate to glycolate; •—•, glyoxylate to oxalate. (A, △), Phosphate buffer (0.1 M); (C, ●), Bicine buffer (0.1 M); (O, ○), Glycine buffer (0.1 M).

(a) The effect of pH on the oxidation of glycolate and glyoxylate is shown in Figure 3A. Tris and Bicine appear to inhibit both activities and the addition of flavin mononucleotide did not activate the enzyme.

(b) The effect of oxalate on the oxidation of glycolate and glyoxylate at pH 8.5 is shown in Figure 4. The lines drawn by regression analysis show partial competitive inhibition. When the data are plotted by the method of Dixon (9), the lines are curved concavely downwards, consistent with partial competitive inhibition. The values for \( K_a \) and \( K_r \) are collected in Table II.

Properties of Lactate Dehydrogenase. The general properties of lactate dehydrogenase from lettuce leaves have been reported by Betsche (1). Here we are concerned with the properties of the enzyme in relation to its possible role in the production of oxalate.

(a) The oxidation of glyoxylate and reduction of NAD can be measured by the increase in \( A \) at 340 nm (Fig. 5). The initial increase in absorbance was followed by a phase in which the absorbance was constant and finally there was a slow decline. We interpret these changes as due to oxalate production and the simultaneous reduction of glyoxylate to glycolate. The decline in absorbance is, we suggest, due to the differential inhibition of the two reactions by oxalate as it accumulates in the cell. A fall in optical density is produced by the addition of oxalate, but we have no explanation for the apparent lag in the inhibition (Fig. 5).

The synthesis of oxalate throughout the period when the optical density was constant or even declining was shown by measuring the formation of \(^{14}C\) oxalate from \(^{14}C\) glyoxylate (Fig. 5).

(b) The effect of pH on the oxidation and reduction of glyoxylate is shown in Figure 6. Oxalate acts as a competitive inhibitor of the oxidation of glyoxylate and a weak noncompetitive inhibitor of glycolate reduction. The values for \( K_a \) and \( K_r \) are given in Table II.

**DISCUSSION**

Although plants can synthesize oxalate from oxaloacetate (3) and ascorbate (16), we are here only concerned with the synthesis from glycolate and glyoxylate. Tolbert's proposal (27) that the formation of oxalate may be due to the lack of substrate specificity of glycolate oxidase is consistent with the properties of the enzyme in a number of cases. For example, the glycolate oxidase of *Oxalis pes-caprae* oxidizes glycolate and glyoxylate at comparable rates and, moreover, the oxidation of glyoxylate is not inhibited by oxalate (17). In other cases, the kinetic properties of glycolate oxidase do not favor a role in the oxidation of glyoxylate. Tolbert (26) has pointed out that peroxisomes are adapted to keep the concentration of glyoxylate low and it follows that a low concentration of glyoxylate will compete only with a high concentration of glycolate for the active site of glycolate oxidase. The rate of glyoxylate oxidation (\( V_2 \)) compared to the rate of glycolate ox-
Fig. 5. Dismutation of glycolate catalyzed by lactate dehydrogenase from lettuce. [1-14C]Glycolate (9,850 dpm \(\mu\)mol) was incubated with NAD (2.75 mg), enzyme and buffer (pH 8.5, Bicine 0.1 M) in a final volume of 1 ml. The change in absorbance at 340 nm was measured in cells of light-path 1 cm and at intervals aliquots were removed, and after the addition of carrier oxalic acid (4 mg), oxalate was isolated. (A), A at 340 nm; (B), \({ }^{14} \mathrm{C}\)oxalate formed with 200 \(\mu\)l enzyme. (C), A at 340 nm; (D), \({ }^{14} \mathrm{C}\)oxalate formed with 100 \(\mu\)l enzyme. At time \(t_{0}\) min, oxalate (10 \(\mu\)mol) was added to a duplicate cell and the absorbance change is indicated by \(x\).

Fig. 6. The effect of oxalate concentration on the oxidation of glycolate to oxalate (A) and the reduction of glyoxylate to glycolate catalyzed by lactate dehydrogenase (B). Activities were assayed as described in "Materials and Methods," except that oxalate was included in the reaction mixture. The data is plotted in the double reciprocal form. The oxalate concentration is indicated: (C), 100 mM; (\(\Delta\)), 25 mM; (\(\bullet\)), 10 mM, (\(\bigcirc\)), control minus oxalate.

\[ \frac{1}{A_{340}} \text{ min}^{-1} = \frac{S_1 + K_m(S_2)}{V_0} \times \frac{1 + S_1}{K_m} \]

For glycolate oxidase from lettuce, the data of Table II and Figure 5 gives

\[ K_m(\text{glycolate}) = 0.33 \text{ mM}; K_m(\text{glyoxylate}) = 9.6 \text{ mM} \]

\[ \frac{V_2}{V_1} = \frac{V_{\text{max}} \text{ for glyoxylate oxidation}}{V_{\text{max}} \text{ for glycolate oxidation}} = \frac{1}{3} \]

For the condition that \(S_1(\text{glycolate}) = 10 \text{ mM and } S_2(\text{glyoxylate}) = 1 \text{ mM, Eq. 1 predicts a ratio of glyoxylate oxidation/glycolate oxidation of 1/870!}

An alternative mechanism of glyoxylate oxidation involves lactate dehydrogenase which catalyzes the reduction of glyoxylate to glycolate and its oxidation to oxalate (Fig. 5). When the \(A\) at 340 nm is constant, the rates of production of glycolate and oxalate must be equal, and the decrease in absorbance occurs when the conversion to glycolate exceeds the synthesis of oxalate—presumably, due to oxalate being a much stronger inhibitor of glyoxylate oxidation \((K_i = 1.4 \text{ mM})\) than of oxalate reduction \((K_i = 105 \text{ mM})\). Such considerations suggest the following model for the controlled production of oxalate from glycolate in lettuce leaves.

![Scheme 1](image-url)

The high activity of the transaminases tends to ensure that the steady-state concentration of glyoxylate in the peroxisomes is low. However, under conditions favoring the rapid production of glyoxylate, or under conditions of nitrogen starvation, glyoxylate may accumulate and leak into the cytosol where lactate dehydrogenase converts it to glycolate and/or oxalate. If the synthesis of oxalate exceeds its rate of transport into the vacuole, oxalate will inhibit its own synthesis and allow lactate dehydrogenase to function as a cytosolic glyoxylate reductase, converting glyoxylate to glycolate which can then reenter the peroxisome. These reactions are those of the glycolate-glyoxylate shuttle proposed by Zelitch (29) and whose operation is claimed to be consistent with the observed incorporation of \(^{3}H\) from \(^{3}H_2O\) into \((R)-[^{3}H]\text{glycolate}\). Presumably, the stereospecificity of leaf lactic dehydrogenase is the same as that of glyoxylate reductase and potato tuber lactic dehydrogenase (7, 14), so that any involvement of lactic dehydrogenase in the glycolate-glyoxylate shuttle cannot be distinguished from the involvement of glyoxylate reductase.

A further aspect of the model deserves comment. It has been proposed (6) that lactate dehydrogenase plays a role in the control of pH in anoxic roots by producing lactate and so lowering the cytosolic pH to a point at which pyruvic decarboxylase becomes active and so shunts the metabolism towards ethanol production. The present model suggests that in some leaves lactate dehydrogenase may function in an oxalate based biochemical pH-stat. Under acid conditions, the production of glycolate from glyoxylate is favored both by the equilibrium of the reaction, glyoxylate +
NADH + H+ → glycolate + NAD+, and the slightly acid pH optimum for this activity (Fig. 3B).

Under alkaline conditions, the production of oxalate is favored both by the equilibrium and the pH optimum. This mechanism may explain the observation that excess cathode uptake in Atriplex sp. leads to enhanced synthesis of oxalate (19).

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